Effects of Transforming Growth Factor-β Released from Gastric Carcinoma Cells on the Contraction of Collagen-Matrix Gels Containing Fibroblasts

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ABSTRACT

To investigate the mechanisms underlying contraction of the stomach wall in cases of gastric scirrhous carcinoma, we have developed an in vitro model for gastric cancer, in which both fibroblasts and gastric carcinoma cells are embedded within a collagen matrix. Gastric carcinoma cells of the scirrhous type (KATO-III) but not the nonscirrhous type (MKN-28) markedly enhanced the ability of human intestine, human lip, and mouse kidney fibroblasts to contract collagen gels. KATO-III cells released transforming growth factor-β (TGF-β) into culture media in an activated form, whereas the MKN-28 cells produced a latent form. The role of TGF-β produced by gastric cancer cells from the scirrhous type was clarified by adding TGF-β (receptor grade) into collagen gels embedded with fibroblasts, contraction being enhanced. Other growth factors tested, including transforming growth factor-α and epidermal growth factor, did not enhance the contraction of collagen gels containing embedded human and rodent fibroblasts. These results suggest that the activated form of TGF-β released from gastric scirrhous carcinoma cells stimulates fibroblasts to contract the collagenous stroma of the stomach wall, which leads to the so-called "linitis plastica" stomach condition.

INTRODUCTION

Scirrhous carcinomas of the stomach presenting as “linitis plastica” have unique clinicopathological features. They arise preferentially in young females, they spread diffusely in the stomach wall without mass formation, and in the terminal stage they cause a rapid contraction of the stomach wall. Because first clinical symptoms only appear after the contraction occurs, the diagnosis of stomach cancer is usually made in the terminal stage, which makes the prognosis of this type of gastric lesion extremely poor (1, 2). An increase in the amount of interstitial collagen in the stomach wall has been reported to be produced extremely poor (1.2). An increase in the amount of interstitial factor, did not enhance the contraction of collagen gels containing em allin cases of gastric scirrhous carcinoma, we have developed an in vitro equivalent to the connective tissue bedded human and rodent fibroblasts. These results suggest that the activated form of TGF-β released from gastric scirrhous carcinoma cells stimulates fibroblasts to contract the collagenous stroma of the stomach wall, which leads to the so-called “linitis plastica” stomach condition.

MATERIALS AND METHODS

Cells and Cell Culture. FLOW-11000 (human embryonic intestine fibroblast), KD (human lip fibroblast), NRK-49F (rat kidney fibroblast), KATO-III (12) (human signet-ring cell carcinoma from scirrhous gastric carcinoma), and MKN-28 (13) (human stomach well-differentiated adenocarcinoma from nonscirrhous gastric carcinoma) were provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). The cells were grown in tissue culture flasks (Corning, Houston, TX) in Dulbecco’s modified minimal essential medium for fibroblasts and RPMI 1640 for gastric carcinoma cells. All culture media were supplemented with 10% FBS, 500 units/ml of penicillin, and 100 μg/ml of streptomycin. Media and their supplements were purchased from GIBCO (Grand Island, NY).

Collagen Gel Culture. Three-dimensional collagen gels were prepared as described by Montesano et al. (14). Briefly, 1 g of type I collagen was extracted from stirred adult rat tail tendons and dissolved in 300 ml of 0.1% acetic acid solution over 48 h at 4°C. After centrifugation at 16,000 × g for 1 h at 4°C, the supernatant was then dialyzed against 0.1% acetic acid solution over 48 h at 4°C. After centrifugation at 16,000 × g for 1 h at 4°C, the supernatant was then dialyzed against 0.1% acetic acid solution over 48 h at 4°C. The dialyzed collagen mixture containing 6.6 × 10⁴ fibroblasts/ml was dispensed into 22-mm plastic dishes (Corning) and allowed to gel for about 10 min at 37°C before the addition of complete culture medium. The gels were then gently detached from the bottom wall of the dishes by passing a 23-gauge needle around the wall perimeter to obtain FCGs including fibroblasts. To assess the effects of scirrhous carcinoma cells on FCG contraction, 0, 10³, and 10⁴ cells/well of KATO-III cells were added to collagen mixtures containing fibroblasts before polymerization. The effects of scirrhous carcinoma cells on FCG contraction in the absence of fibroblasts were also examined. Nonscirrhous gastric carcinoma cells (MKN-28) were cultured in FCG as a control for KATO-III cells. To determine the effects of TGF-β on gel contraction, various concentrations of TGF-β from porcine platelets (R&D System, Inc., Minneapolis, MN) were added to control media of FCGs along with fibroblasts. In the same way, the effects of TGF-α and EGF were also examined. Culture media (RPMI 1640 with 10% FBS) and growth factors were renewed every 48 h. Gel contraction was quantified at 24-h intervals by placing the dishes on transparent metric-scale graph paper and measuring the major and minor axes of the collagen gels. The gel areas were calculated from the average diameter and expressed as a percentage of the initial area.

Bioassay for TGFs. As a possible candidate for the factor inducing gel contraction, TGF activity was examined in CM containing KATO-III or MKN-28 cells. Growth was in complete RPMI 1640 in 75-cm² tissue culture dishes, and after the cultures had become confluent, they were rinsed twice with Ca²⁺- and Mg²⁺-free Hanks’ balanced salt solution and then incubated in serum-free RPMI 1640 for 1 h. Subsequent to changing the medium again, the cultures were incubated...
overnight. From this time onward media were collected every 2 days for up to 10 days, centrifuged to remove cells and cell debris, and then divided into two parts. Half volumes of CM were acidified to pH 2.0 with 1 N HCl, left for 1 h at 4°C, neutralized to pH 7.5 with 1 N NaOH, by which the latent form of TGF-β is converted to the active form (15), and then frozen and kept at −20°C. The other CM portions were frozen without activation. The protein was concentrated 10-fold with an Amicon YM-2 membrane, and the protein concentration of the CMs was determined by dye-binding assay (16). Activities of TGF-β and TGF-α in CMs were determined by bioassay using NRK-49F cells (10³ cells/well) as the indicator cells with and without the addition of 1 ng/ml of EGF (Biomedical Technologies Inc., Stoughton, MA). The soft agar medium was prepared according to the method of DeLarco and Todaro (17). After 10 days incubation, colonies of >20 cells (0.075 mm diameter) were counted using an inverted microscope. The number of colonies in the absence or presence of EGF is indicative of TGF-α or TGF-β activity, respectively (17).

RESULTS

KATO-III cells derived from a scirrhous carcinoma markedly enhanced the contraction of FCGs by embedded fibroblasts in a cell number-dependent manner (Figs. 1A and 2). Although each fibroblast by itself induced a progressive contraction of FCG in the presence of serum (Fig. 2; ○) as previously reported (6-10), the rate of contraction depended on the numbers of KATO-III cells contained (Fig. 1A: 10⁴ and 10⁵ cells/well; Fig. 2: △ and O). The rate of gel contraction without KATO-III cells was different for each fibroblast used, although in all cases an enhancing effect of KATO-III cells on the contraction was clear. FLOW 11000, NRK-49F, and KD by themselves contracted the gels to 58, 82, and 46%, respectively, of their initial areas by day 5, whereas adding 10⁵ KATO-III cells caused contraction to 40, 47, and 26%, respectively, by day 5 (Fig. 2). FCGs including fibroblasts placed in wells containing 10⁵ KATO-III cells also contracted in the same manner as those including both fibroblasts and KATO-III cells, suggesting that the contraction of FCGs was induced by a humoral interaction between KATO-III cells and the fibroblasts. The gel contraction was associated with a change in the appearance of collagen discs from translucent to opaque and dense. No contraction was found in FCGs without fibroblasts, irrespective of the presence of KATO-III cells (data not shown). MKN-28 cells derived from a nonscirrhous adenocarcinoma did not enhance the contraction of FCGs by included FLOW-11000, NRK-49F, or KD fibroblasts, i.e., no differences were found in the contraction of gels with or without MKN-28 cells (Figs. 1B and 3).

Since the contraction of collagen gels with fibroblasts has been reported to require the presence of TGF-β (10), we measured TGF-β activity in the CM using a colony-forming assay in soft agar with NRK-49F cells (Table 1). In Table 1, the columns
IN VITRO MODEL FOR SCIRRHOUS CARCINOMA

Fig. 3. Kinetics of FCG contraction in the MKN-28 cell case. The experimental conditions were the same as those explained for Fig. 2 except that MKN-28 cells were applied instead of KATO-III cells. No significant differences are evident between controls and gels containing MKN-28 cells at any time point.

Table 1 TGF activities in conditioned media of the gastric carcinoma cell lines

<table>
<thead>
<tr>
<th>Effector</th>
<th>Concentration</th>
<th>Colonies/well*</th>
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| TGF-β             | 1 ng/ml       | 0    | 54 ± 3
| CM (KATO-III)     | 40 µg/ml      | 13 ± 1| 41 ± 3
| Untreated         | 40 µg/ml      | 14 ± 3| 43 ± 4
| Acid neutralized  | 40 µg/ml      | 0    | 3 ± 1
| CM (MKN-28)       | 40 µg/ml      | 0    | 32 ± 2
| Untreated         | 40 µg/ml      | 0    | 32 ± 2

*One thousand NRK-49F cells/plate in 0.3% agar medium containing 6% fetal calf serum. Protein from conditioned medium (final concentration, 40 µg/ml) was added to each plate in the presence or absence of 1 ng/ml EGF.

**Mean ± SD of quadruplicate plates.

"-EGF" and "+EGF" give TGF-α and TGF-β activities, respectively, based on this definitive property. CM of KATO-III cells showed a 13-fold greater activity for TGF-β than those of MKN-28 cells using CM without acid-neutralized treatment, whereas after the activation by acidification, the CM with MKN-28 cells, but not with KATO-III cells, demonstrated increased TGF-β activity. These results indicate that KATO-III cells secrete TGF-β in an activated form, while, MKN-28 cells mainly produce a latent form. KATO-III cells also contained TGF-α activity (Table 1, -EGF).

The effects of TGF-β on gel contraction are illustrated in Figs. 1C and 4. FCGs with FLOW-11000, NRK-49F, and KD in control medium contracted to 52, 38, and 37%, respectively, of their initial areas by day 5 (Fig. 4, ●), whereas, in the presence of 1 ng/ml of TGF-β, they contracted to 29, 15, and 17%, respectively (Fig. 4, ○). Earlier we reported that KATO-III cells and MKN-28 cells proliferate in collagen gels (18), which indirectly suggested that each cell line could secrete TGFs into collagen gels accompanied by DNA and protein synthesis during the cell cycle. The numbers of fibroblasts contained in collagen gels showed no significant change during the experimental period whether in the presence of TGF-β or not (data not shown). This indicated that the enhanced gel contraction in the presence of TGF-β did not result from a proliferation of fibroblasts induced by this factor. TGF-β could stimulate gel contraction even in the absence of serum (Fig. 5). Without serum (1 mg/ml of bovine serum albumin added), no contraction of FCGs was observed for up to 7 days, whereas the presence of 5 ng/ml of TGF-β caused a marked contraction. On the other hand, EGF (4 and 40 ng/ml) and TGF-α (0.5 and 5 ng/ml) showed neither contraction of FCGs nor influence on the contraction induced by TGF-β (Fig. 6).

DISCUSSION

Most cells in multicellular organisms are in contact with an intricate extracellular matrix network, which controls various functions of normal (5, 19, 20) and malignant (21, 22) cells. Differentiation of normal mammary cells (5, 19, 20) cultured in floating collagen gels is well known, which makes this method an appropriate model in vitro to demonstrate probable cell functions in vivo. In scirrhous carcinoma of the stomach,
cancer cells exist in a collagen-rich stroma along with fibroblasts. We report here a new in vitro model for the stomach wall of scirrhous carcinomas using the collagen gel culture technique and give one possible answer to the question of what is the mechanism underlying contraction of the stomach wall in this disease. The coculture of normal cells with malignant cells in collagen gels has not been previously tried, although various kinds of growth factor, i.e., TGF-β, TGF-α, EGF, and platelet-derived growth factor (35–38). In this experiment, we observed TGF-α and TGF-β activities in conditioned medium but did not examine whether TGF-β reacted as an autocrine growth inhibitor to gastric carcinoma cells. However KATO-III cells lack an autocrine growth inhibition loop for TGF-β because they can still grow in the presence of activated TGF-β secreted by themselves. In an analysis of TGF-β mRNA levels in surgical specimens (38), 90% of the scirrhous gastric carcinomas exhibited higher levels of TGF-β mRNA than corresponding normal tissues. In nonscirrhous gastric carcinomas, in contrast, TGF-β mRNA could be detected, but the TGF-β polypeptide was not demonstrated by Western blot analysis due to an extremely low level of protein synthesis. TGF-β is known to exist in a latent form in extracts of cells and tissues (15, 39). This latent TGF-β consists of active TGF-β and glycoproteins of about M, 400,000 linked by noncovalent bonds (40). TGF-β activity has been detected in the ethanol extraction of cells and tissues after acidification because the latent form is activated by treatment with acid, urea, or heat in vitro (41). In the present study, a part of the CM was not acidified, allowing comparative analysis of only the active form of TGF-β by colony-forming ability of NRK-49F cells. No activity of TGF-β was detected in the CM of MKN-28 cells before acidification, although after acidification TGF-β activity expression was as high as with KATO-III cells. The mechanisms of activation of TGF-β secreted by KATO-III cells, lacking other normal and cancer cells, are unknown. KATO-III cells also possibly secrete an activating factor for latent TGF-β or mutated masking components. The recognition site of TGF-β by the anti-TGF-β antibody (R&D System) in Western blot analysis (38) may be masked by binding protein in nonscirrhous gastric carcinoma. Our result clearly demonstrated that gastric carcinoma cells of both scirrhous and nonscirrhous types secrete TGF-β, although in one case this is activated and in the other it is in its latent form. It is still a matter of argument whether nonscirrhous carcinomas secrete TGF-β polypeptides (38). We could detect TGF-β activity in other cell lines derived from nonscirrhous carcinomas after acidification (data not shown), but further experiments are necessary to clarify this point.

TGF-β affects many different cell types in ways unrelated to control of proliferation (31–34). Fibroblasts incorporated into collagen gels are known to cause a progressive contraction of the gels in the presence of TGF-β in serum (6–10). Our results suggested that these phenomena occur not only during wound healing (8, 10) but in the progression of gastric carcinomas. Under the same experimental conditions, TGF-α and EGF did not significantly stimulate contraction of collagen gels, suggesting that this activity of TGF-β is not a general property of growth factors. While the mechanisms underlying this phenomenon are not known in detail, they may be related to the effects of TGF-β stimulating linkage between collagen fibers or fibronectin receptors on the fibroblast cell membranes by increasing fibronectin (31), or receptors themselves (32), or by stimulating intracellular actin formation related to cell contraction (42).

Contributions of platelets (40), macrophages (43), and activated lymphocytes (44) must also be considered as sources of TGF-β in scirrhous carcinoma.

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